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Iron-catalyzed Autoxidation of Cholesterol in the Presence of Unsaturated Long-chain Fatty Acid[1)]

Toshiki Muto, Jun Tanaka, Toshiaki Miura, and Michiya Kimura*

Faculty of Pharmaceutical Sciences, Hokkaido University, Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo 060, Japan

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Oxy-functionalization of cholesteryl acetate (**1a**) occurred, giving 3β-acetoxy-5,6-epoxycholestane (**2a**), 3β-acetoxycholest-5-en-7-one (**3a**), 3β-acetoxycholest-5-en-7-ol (**4a**), and an unidentified product (**5**), when **1a** was oxidized by a system consisting of $Fe(acac)_3$ and the hydroperoxide of an unsaturated long-chain fatty acid (LH) such as oleic, linolic, or linolenic acid (Table I). The epoxidation of stilbene by the same system was found to be non-stereospecific. These results and the fact that the reaction in this system was inhibited by a radical scavenger (BHT) were fairly compatible with those obtained with the $Fe(acac)_3$-^{t}BuOOH system, which is assumed to generate oxy and peroxy radicals.

Autoxidation of **1a** and cholesterol (**1b**) in the presence of $Fe(acac)_3$ and LH proceeded after a time-lag of several hours and was also inhibited by BHT. The marked stereoselectivity of β-epoxidation ($\beta/\alpha+\beta=0.72$) and the extent (about 30—40%) of allylic oxidation in the autoxidation were in fair agreement with those found for **1a** in the $Fe(acac)_3$-LOOH system (Table II). Autoxidation of stilbene in the presence of $Fe(acac)_3$ and LH also led to non-stereospecific epoxidation. Thus, the autoxidation of cholesterols (**1a** and **1b**) in the $Fe(acac)_3$-LH system was assumed to be a radical reaction in which LOO· and LO· are the attacking species.

Keywords——autoxidation; cholesterol; co-oxidation; epoxidation; hydroperoxide; lipid peroxidation; radical pathway; stilbene; tris(acetylacetonato)iron(III); unsaturated long-chain fatty acid

The biological oxidation of cholesterol has become of interest in recent years in relation to mutagenicity,[2a)] skin carcinoma,[2b)] angiotoxicity,[2c)] cytotoxicity,[2d)] and stimulation and inhibition of enzyme systems.[2d)] In the microsomal lipid peroxidation system, cholesterol was reported to be oxidized by the radical species generated from the lipid peroxide in the presence of iron-adenosine diphosphate (ADP) complex.[3a,b)] Alkyl peroxy and alkoxy radicals of fatty acids are assumed to be generated in biological lipid peroxidation.[4a)] We reported the allylic oxidation and epoxidation of cholesteryl acetate by a mixture of *tert*-butyl hydroperoxide (^{t}BuOOH) and tris(acetylacetonato)iron(III), $Fe(acac)_3$, and proposed the participation of ^{t}BuOO· and ^{t}BuO· radicals as the attacking species in these reactions.[5)] The observed β-stereoselectivity in the epoxidation of cholesteryl acetate by the ^{t}BuOOH-iron chelate system was in good accordance with that seen with the lipid peroxidation system in cell-free preparations of rat liver.[6)] These results prompted us to investigate the iron-catalyzed reaction of cholesteryl acetate with the hydroperoxides of fatty acids, which are essential components of the usual bodyl lipids. In this paper, we describe firstly the oxy-functionalization of cholesteryl acetate by hydroperoxides of several unsaturated long-chain fatty acids and $Fe(acac)_3$ and secondly the co-oxidation of cholesterol and its acetate during the autoxidation of these acids, also catalyzed by the iron-chelate.

Results and Discussion

Oxy-functionalization of Cholesteryl Acetate by the Fatty Acid Hydroperoxide-$Fe(acac)_3$ System

A benzene solution of cholesteryl acetate (**1a**), oleic acid hydroperoxide, and $Fe(acac)_3$ was heated at 60°C for 24 h under an Ar atmosphere. The hydroperoxide was consumed

rapidly in the early period of the reaction and then more slowly with increasing oxidation of **1a**, as shown in Fig. 1. The products were 3β-acetoxy-5,6-epoxycholestane (**2a**, epimeric mixture), 3β-acetoxycholest-5-en-7-one (**3a**), 3β-acetoxycholest-5-en-7-ol (**4a**, epimeric mixture), and an unidentified compound (**5**),[7a] as summarized in Table I. The reaction, on the other hand, was retarded by adding a radical scavenger, 2,6-di-*tert*-butyl *p*-cresol (BHT). These results were identical with those observed in the reaction of **1a** with the Fe(acac)$_3$-^{t}BuOOH system, where the attacking species are assumed to be ^{t}BuOO· and ^{t}BuO· radicals.[5] The epoxidation of stilbene was found to be non-stereospecific, when the reaction proceeds by a radical mechanism and thus free rotation around the attacked C–C bond can occur in the intermediate.[8] Under the conditions of the present reaction, both *trans*- and *cis*-stilbenes gave an isomeric mixture of epoxide in an approximate ratio of *trans*: *cis*=95: 5, as was also observed in their epoxidation by the Fe(III)-^{t}BuOOH system.[5] It may, therefore, be reasonable to assume that the Fe(acac)$_3$-fatty acid hydroperoxide (LOOH) system produces LOO· and LO· radicals which then attack **1a** to give the oxygenated products, **2a**, **3a**, and **4a**.

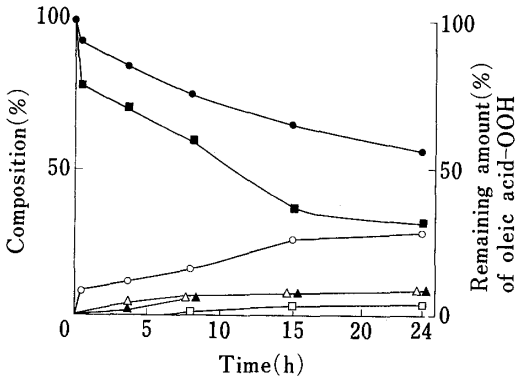

Fig. 1. Time Course of Oxidation of Cholesteryl Acetate with Fe(acac)$_3$-Oleic Acid Hydroperoxide

—●—: **1a**, —○—: **2a**, —△—: **3a**, —▲—: **4a**, —□—: **5**, —■—: oleic acid hydroperoxide.

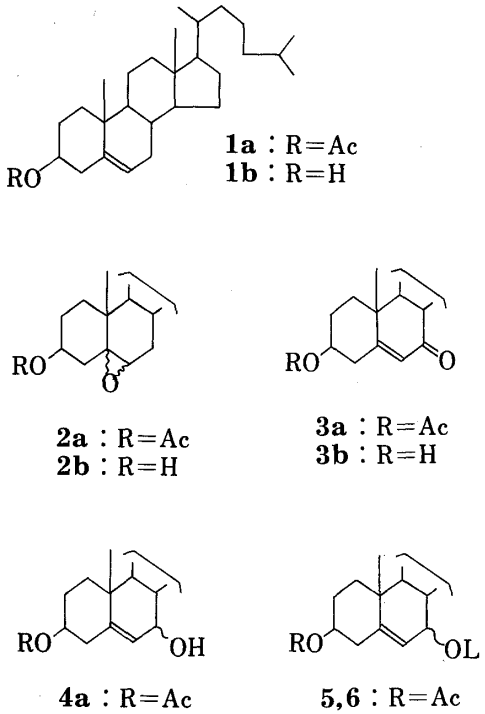

Chart 1

Table I. Oxidation of Cholesteryl Acetate with Fe(acac)$_3$ and LOOH[a]

LOOH	Fe(acac)$_3$	Conversion (%)	Yield (%)				Allylic oxidation (%)
			2a[b]	3a	4a	5	
Oleic acid-OOH	−	10	6	1	1	2	40
	+	50	34 (0.72)	6	6	4	32
Linoleic acid-OOH	−	9	5	2	1	1	44
	+	46	32 (0.72)	5	5	4	30
Linolenic acid-OOH	−	5	3	1	0	1	40
	+	14	8	3	1	2	43

a) Data are the means of three runs.
b) Numbers in parentheses are ratios of β-epoxide to the total epimers formed.

The hydroperoxides of linoleic as well as linolenic acids similarly oxygenated **1a**, as summarized in Table I. The epoxidation, which may be brought about by the peroxy radicals,[5] was highly β-stereoselective ($\beta/\alpha+\beta=0.72$) in the reactions using these hydroperoxides, as shown in Table I and as observed in the reactions of **1a** with the Fe(III)-^{t}BuOOH system.[5] The hydroperoxides (LOOH) of unsaturated long-chain fatty acids are generally considered to be unstable, in contrast to the usual ROOH such as *tert*-butyl and cumenyl hydroperoxides. The reaction using LOOH proceeded, to some extent, in the absence of the iron catalyst, as shown in Table I. The assisted homolysis of hydroperoxides, in which radicals are produced at greatly enhanced rates, will be favored in the cases of LOOH as an acid.[4b] Thus, the results shown in Table I may be an indication that the hydroperoxides LOOH are liable to be autocatalytically decomposed into LOO· and LO· radicals.

Autoxidation of Cholesteryl Acetate in the Presence of Fatty Acid and Fe(acac)$_3$

Autoxidation of cholesteryl acetate (**1a**) was carried out in benzene solution containing Fe(acac)$_3$ and an unsaturated long-chain fatty acid (LH) such as oleic, linoleic, or linolenic acid, at 60°C. The oxidation products of **1a** were the epoxide (**2a**), C(7)-ketone (**3a**), C(7)-alcohol (**4a**), and unidentified compounds (**6**).[7b] The stereoselectivity ($\beta/\alpha+\beta=0.72$) of epoxidation and the extent (31—42%) of allylic oxidation were in fair agreement with those in the reaction of **1a** with the Fe(acac)$_3$-LOOH system (Table I), as summarized in Table II. The autoxidation did not occur in the absence of the iron chelate, the unsaturated fatty acid (LH), or both of them, or when a saturated fatty acid such as stearic acid was employed instead of LH.

The time course of the reaction profile was followed by analysis of the starting materials and the products. One of the starting materials, LH, began to decrease after a time lag of two hours and then the oxidation of the other one, **1a**, proceeded with decrease of LH, as shown in Fig. 2. From these results and the fact that the unsaturated long-chain fatty acids (LH) can give the corresponding hydroperoxides in an autoxidation system,[4b] the reaction of **1a** with molecular oxygen in the presence of LH and Fe(acac)$_3$ seemed to proceed homolytically. In fact, the reaction was hindered by added BHT, as shown in Fig. 3. The epoxidation of stilbene also occurred in the autoxidation system containing oleic acid and the iron chelate and gave an isomer ratio of *trans*: *cis*=96: 4. Therefore, the autoxidation of **1a** in the Fe(acac)$_3$-LH system is assumed to be a radical reaction, where LOO· and LO· are the attacking species, as in the Fe(acac)$_3$-LOOH system described above.

Table II. Fe(acac)$_3$-Catalyzed Autoxidation of Cholesteryl Acetate and Cholesterol in the Presence of LH[a]

LH	R[c]	Fe(acac)$_3$	Conversion (%)	Yield (%)				Allylic oxidation (%)
				2[b]	3	4	6	
Oleic acid	Ac	−	0					
		+	38	22 (0.71)	6	9	1	42
	H	−	0					
		+	16	9 (0.73)	5	2		44
Linoleic acid	Ac	−	1	1	0	0	0	0
		+	36	25 (0.72)	4	5	2	31
	H	−	11	11 (0.70)	0	0		0
		+	51	32 (0.72)	9	10		37
Linolenic acid	Ac	−	0					
		+	38	27 (0.72)	4	7	1	38
	H	−	0					
		+	33	22 (0.68)	7	4		33

a) Data are the means of three runs.
b) Numbers in parentheses are ratios of β-epoxide to the total epimers formed.
c) Ac: cholesteryl acetate, H: cholesterol.

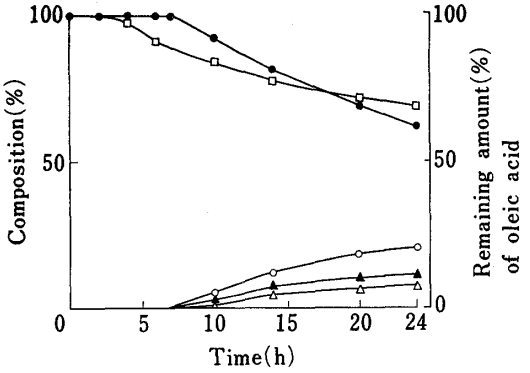

Fig. 2. Time Course of $Fe(acac)_3$-Catalyzed Autoxidation of Cholesteryl Acetate in the Presence of Oleic Acid

—●—: **1a**, —○—: **2a**, —△—: **3a**, —▲—: **4a**, —□—: oleic acid.

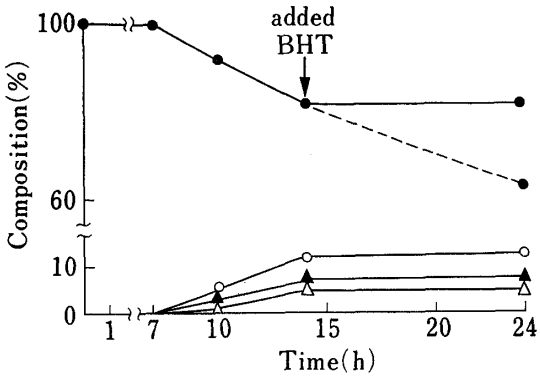

Fig. 3. Effect of BHT on the Oxidation

—●—: **1a**, —○—: **2a**, —△—: **3a**, —▲—: **4a**, --●--: **1a** without BHT.

Autoxidation of Cholesterol in the $Fe(acac)_3$-LH System

The autoxidation of cholesterol (**1b**) in the presence of $Fe(acac)_3$ and the unsaturated long-chain fatty acid (LH) similarly gave the epoxide (**2b**), C(7)-ketone (**3b**), and C(7)-alcohol (**4b**). The epimer ratios ($\beta/\alpha+\beta$) of **2b** thus obtained were found to be between 0.68 and 0.73, again showing a marked β-stereoselectivity. The allylic oxidation of **1b** was similar in extent (33—47%) to that of **1a**, as summarized in Table II. The autoxidation of **1b** in this system is, therefore, also assumed to be a radical reaction.

From these results, the mechanism of the above-mentioned reactions of **1a** and **1b** may be rationalized on the basis of the scheme shown in Chart 2.

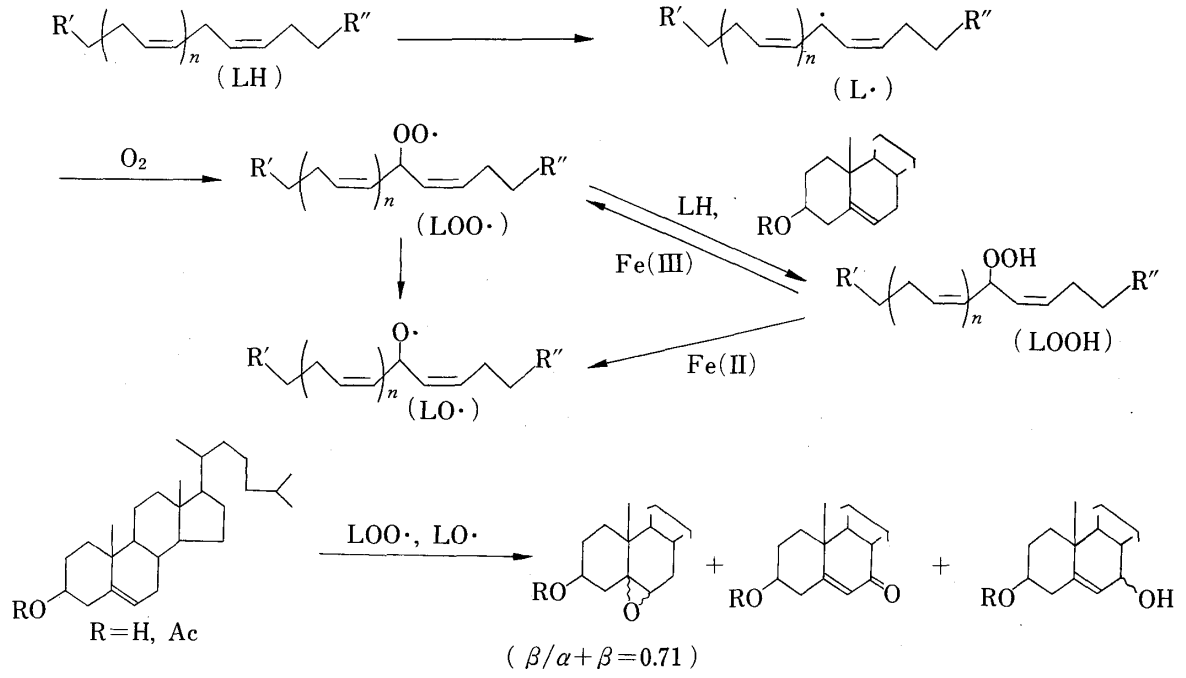

Chart 2

The attacking species was reported to be neither singlet oxygen, $^1O_2$, nor superoxide anion, $O_2^{\cdot-}$, in the oxidation of cholesterol by the biological lipid peroxidation system.[3a,c] Such an oxidation of cholesterol was pointed out to be closely related to the peroxidation of lipids

and is a radical reaction catalyzed by metals, particularly iron complexes.[3d,6] A marked β-stereoselectivity of the epoxidation was observed in the reaction by the $Fe(acac)_3$-LH-O_2 system as described above, and is reasonably consistent with that given by the lipid peroxidation system in cell-free preparations of rat liver.[6] The oxidation of cholesterol in such a biological system may, therefore, involve the oxy and peroxy radicals of the unsaturated acyl moieties in the lipid molecules.

Experimental

General Methods——Melting points were taken on a micro hot-stage apparatus and are uncorrected. Preparative thin-layer chromatography was carried out on silica gel (Wako gel B5F) by using the solvent system of Ph/AcOEt (19: 1). Detection was achieved with 10% H_2SO_4, with heating at about 130°C for 5 min. Gas chromatograms were taken on a Shimadzu GC-4CM PF machine. Proton magnetic resonance (PMR) spectra were measured with a JEOL JNM-FX 100FT spectrometer at 100 MHz with tetramethylsilane as an internal standard in $CDCl_3$. Determination of the product distribution and the epimeric ratio of the epoxide in the reaction mixture were carried out by the reported methods using an Iatron TFG-10 Thinchrograph (TLC-FID) and PMR spectrometer, respectively.[9]

Materials——$Fe(acac)_3$ (mp 180°C, lit.[10a] 182°C; Dozin Yakkagaku Lab.), BHT (Wako Pure Chem. Ind. Ltd.), *trans*- and *cis*-stilbenes (Aldrich Chem. Company, Inc.), oleic and stearic acids (Wako Pure Chem. Ind. Ltd.), linoleic, linolenic, and *n*-pentadecanoic acids (Tokyo Kasei Co. Ltd.) were obtained commercially and used without purification. Cholesterol (**1b**; Wako Pure Chem. Ind. Ltd.) was purified by recrystallization from EtOH–MeOH, mp 149.5°C, lit.[10b] 149°C. All authentic specimens of 5,6-epoxy-cholestan-3β-ol (**2b**), 3β-hydroxycholest-5-en-7-one (**3b**), cholest-5-ene-3β,7-diol (**4b**), their acetates (**2a**, **3a**, **4a**), and cholesteryl acetate (**1a**) were prepared as described in the previous papers[11,12] and purified by the ordinary method. Hydroperoxides of oleic, linoleic, and linolenic acids were prepared by photo-sensitized peroxidation of the corresponding fatty acid.[13] Their purities were determined by iodometry to be 72, 70, and 68%, respectively.

Oxidation of Cholesteryl Acetate (1a) with $Fe(acac)_3$ and Hydroperoxide (LOOH)——1. Time Course of the Reaction Profile (Fig. 1): A benzene solution (10 ml) of **1a** (100 mg, 0.23 mmol), $Fe(acac)_3$ (10.3 mg, 2.83×10^{-2} mmol), and oleic acid hydroperoxide (630 mg, 2.0 mmol) was heated at 60°C for 24 h under an Ar atmosphere. At each specified time, 0.2 ml of the reaction mixture was sampled and diluted with ether (2 ml). The ether solution was washed with 2 N KOH, 2 N HCl, sat. aq. $NaHCO_3$, and sat. aq. NaCl successively, then dried over anhydrous Na_2SO_4, and filtered. The filtrate was concentrated under a stream of N_2 and subjected to thin layer chromatography–flame ionization detector (TLC–FID) for determination of the product distribution. At the specified time, a further 0.15 ml of the reaction mixture was sampled and diluted with EtOH; a portion of this solution was then assayed by the glutathione peroxidase method[14] to determine the amount of the hydroperoxide remaining.

2. Oxidations using Hydroperoxides of Various Fatty Acids (Table I): A benzene solution (2.5 ml) of **1a** (25 mg, 5.8×10^{-2} mmol), $Fe(acac)_3$ (2.10 mg, 5.9×10^{-3} mmol), and LOOH (180 mg, 7.5×10^{-1} mmol) was heated at 60°C for 24 h under an Ar atmosphere. The reaction mixture was worked up as described above. The consumption of **1a**, product distribution, and the epimer ratio of the epoxide were determined according to the general methods.[9]

3. Effect of Radical Scavenger on the Reaction: A benzene solution (2.3 ml) of **1a**, $Fe(acac)_3$, and oleic acid hydroperoxide in the same amounts as described above was treated in the presence of various amounts of BHT (2.3×10^{-3} M, 5.8×10^{-3} M, 1.2×10^{-2} M, 1.9×10^{-2} M, and 2.3×10^{-2} M). The consumption of **1a** and the product distribution were determined by the TLC–FID method after usual work-up.

$Fe(acac)_3$-Catalyzed Autoxidation of Cholesterol (1b) and Its Acetate (1a) in the Presence of Fatty Acid (LH)——1. Time Course of the Reaction Profile (Fig. 2): A benzene solution (10 ml) of **1a** (100 mg, 0.23 mmol), $Fe(acac)_3$ (9.76 mg, 2.76×10^{-2} mmol), and oleic acid (725 mg, 2.31 mmol) was heated at 60°C, and O_2 was bubbled through the solution. At each specified time, 0.1 ml of the reaction mixture was sampled, diluted with ether (2 ml), and extracted 5 times with 0.5 ml of 2 N KOH. The organic layer was subjected to TLC–FID for determination of product distribution after work-up as mentioned above. The alkaline layer was, on the other hand, acidified with 2 N HCl, saturated with NaCl, and extracted with ether (0.5 ml × 5). The ether layer was washed with sat. aq. NaCl, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue thus obtained was re-dissolved in ether (0.5 ml) containing *n*-pentadecanoic acid as an internal standard, methylated with CH_2N_2, and subjected to gas–liquid chromatography (GLC) to determine the amount of oleic acid remaining. GLC conditions: glass column (1.5% SE30, 3 mmφ × 2 m), temp. (220°C for injection, 190°C for column, 220°C for detector), carrier gas (N_2 50 ml/min), relative retention time (methyl *n*-pentadecanoate: 1.00, methyl oleate: 2.57), relative sensitivity (methyl *n*-pentadecanoate: 1.00, methyl oleate: 0.747).

2. Autoxidations in the Presence of Various LH (Table II): A benzene solution (2.5 ml) of **1a** or **1b**

(25 mg, 5.8×10^{-2} mmol), $Fe(acac)_3$ (2.10 mg, 5.90×10^{-3} mmol), and LH (180 mg, 7.5×10^{-1} mmol) was bubbled through with O_2 at 60°C for 24 h. After work-up of the reaction mixture as described above, the consumption of **1a** or **1b** and the product distribution were determined according to the general method.

No reaction was found to occur with stearic acid, in contrast to the unsaturated fatty acids.

3. Effect of Radical Scavenger on the Reaction (Fig. 3): A benzene solution of **1a**, $Fe(acac)_3$, and oleic acid in the same amounts as described above was bubbled through with O_2 at 60°C for 24 h in the presence of BHT (3.2 mg, 1.5×10^{-2} mmol). No reaction occurred.

A mixture of **1a**, $Fe(acac)_3$, oleic acid, and benzene in the same amounts as cited above was similarly treated for 14 h, then BHT (15.1 mg, 6.8×10^{-2} mmol) was added to the reaction mixture, and O_2 was bubbled through the solution for another 10 h at 60°C. At each specified time, 0.1 ml of the reaction mixture was sampled and worked up as described above for determination of the product distribution.

Epoxidation of Stilbene——A benzene solution (5 ml) of *trans*-stilbene or the *cis*-isomer (40 mg, 2.2×10^{-1} mmol), $Fe(acac)_3$ (4.81 mg, 1.36×10^{-2} mmol), and oleic acid hydroperoxide (360 mg, 1.5 mmol) was heated at 60°C for 24 h under an Ar atmosphere. After usual work-up, the yield and the isomer ratio of the epoxide were determined by the TLC–FID and proton magnetic resonance (PMR) methods, respectively, as previously reported.[12]

A benzene solution (5 ml) of stilbene (40 mg, 2.2×10^{-1} mmol), $Fe(acac)_3$ (4.81 mg, 1.36×10^{-2} mmol), and oleic acid (360 mg, 1.5 mmol) was treated as described above. The yield and the isomer ratio of the epoxide were determined by the reported method.[12]

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